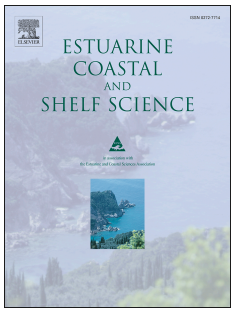
Accepted Manuscript



Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite)

Luísa Magalhães, Xavier de Montaudouin, Simão Correia, Guillemine Daffe, Patrice Gonzalez, Etelvina Figueira, Jorge M.S. Gonçalves, Rosa Freitas

PII: S0272-7714(18)30350-0

DOI: https://doi.org/10.1016/j.ecss.2019.02.012

Reference: YECSS 6107

To appear in: *Estuarine, Coastal and Shelf Science*

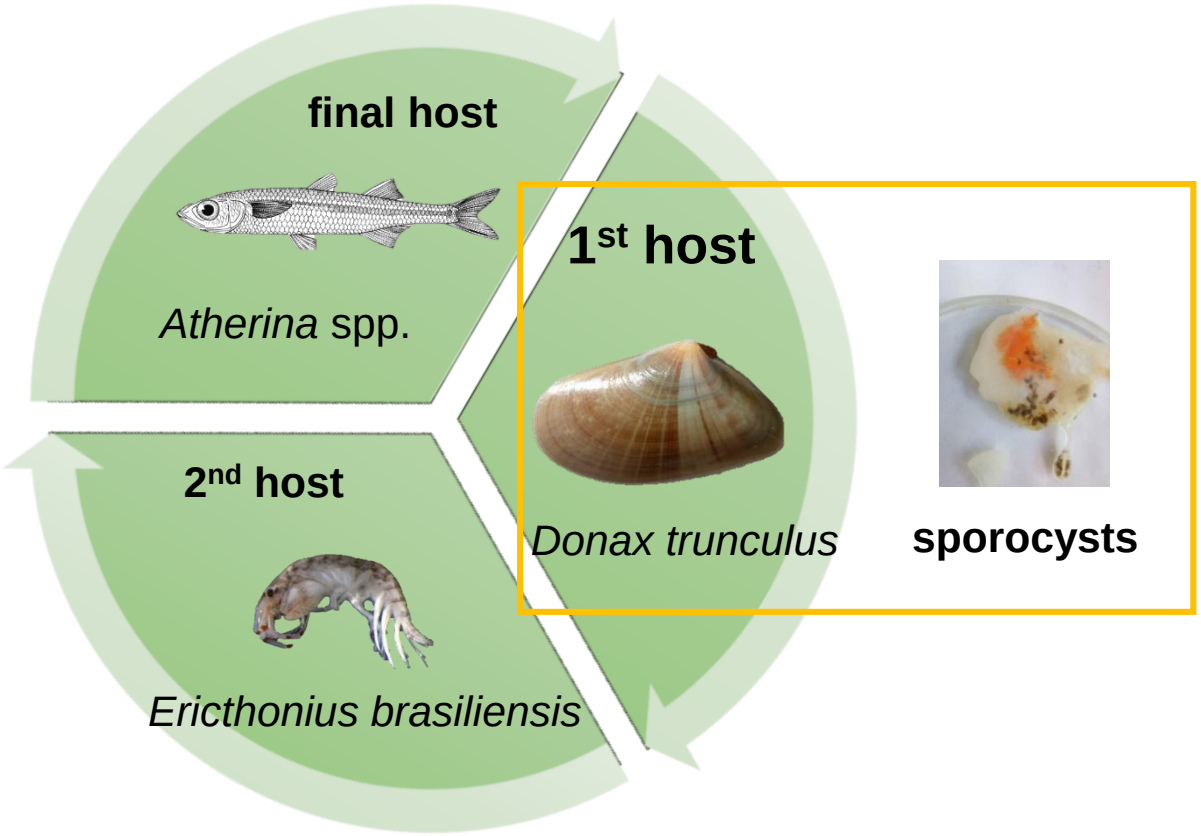
Received Date: 25 April 2018

Revised Date: 15 January 2019

Accepted Date: 3 February 2019

Please cite this article as: Magalhães, Luí., de Montaudouin, X., Correia, Simã., Daffe, G., Gonzalez, P., Figueira, E., Gonçalves, J.M.S., Freitas, R., Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite), *Estuarine, Coastal and Shelf Science* (2019), doi: https://doi.org/10.1016/j.ecss.2019.02.012.

Bacciger bacciger life cycle



- oxidative stress response
- cell metabolism
- energy demand

Seasonal variation of transcriptomic and biochemical parameters of *Donax trunculus* related to its infection by *Bacciger bacciger* (trematode parasite)

Magalhães, Luísa[a,b], de Montaudouin, Xavier[b], Correia, Simão[a], Daffe, Guillemine[c], Gonzalez, Patrice[b], Figueira, Etelvina[a], Gonçalves, Jorge M. S.[d], Freitas, Rosa[a*]

[a] Univ. Aveiro, Departamento de Biologia & CESAM, 3810-193 Aveiro, Portugal

[b] Univ. Bordeaux, CNRS, UMR EPOC, Station Marine, 2, rue du Pr Jolyet, F-33120 Arcachon, France

[c] Univ. Bordeaux, CNRS, Observatoire Aquitain des Sciences de l'Univers, UMS 2567 POREA, F-33615, Pessac, France

[d] Univ. Algarve, Centro de Ciências do Mar - CCMAR, FCT, Campus de Gambelas, 8005-139 Faro, Portugal

[*] Corresponding author: Tel.: +351 234 370 350; fax. +351 234 372 587

E-mail address: rosafreitas@ua.pt

Abstract

The wedge clam (*Donax trunculus*) is widely distributed along exposed Atlantic coasts, from France to Senegal. This species has high commercial importance, with the mean capture production on the last ten years of approx. 850 tonnes (50% represented by Portugal captures). *D. trunculus* populations are modulated by several drivers such as tidal range, temperature, sediment grain size, fishing pressure, predation and parasitism. Regarding parasitism, *D. trunculus* is the first intermediate host of *Bacciger bacciger* (trematode parasite) where the sporocysts develop. The sporocyst is the most damaging stage, reported as being responsible for bivalve castration and flesh mass depletion. In order to test the hypothesis that *B. bacciger* infection modified wedge clam health status, including its biochemical performance and gene expression, clams were sampled every other month during one year in the Faro

beach (south coast of Portugal). The results obtained revealed that *B. bacciger* total prevalence ranged between 0 and 33% in May and July, respectively. Overall, transcriptomic and biochemical results showed that *B. bacciger* induced in *D. trunculus* defence mechanisms against oxidative stress and increased the host metabolism and energy demand, especially in spawning and spent periods. The present work concluded that the markers used can provide additional and ecologically relevant information not only about the environmental conditions that animals experience but also the invasion effects of pathogens. These findings can contribute to predicting organism chance of reproduction and survival in their natural context, which can be applied in bivalve conservation and disease episodes management.

Keywords

Bivalves; Host-parasite system, Gene expression; Biochemical markers; oxidative stress; metabolism.

1. INTRODUCTION

The wedge clam, *Donax trunculus* Linnaeus, 1758 (Bivalvia: Donacidae), is a warm-temperate species distributed from the Atlantic coast of France to Senegal (Tebble 1966) and the shallow bottoms of the Black Sea, Mediterranean Sea (Bayed and Guillou 1985) and Marmara Sea (Deval 2009). The species natural beds occur primarily in the intertidal zone, from 0 to 6 m (Gaspar et al. 2002), in highly energetic environments where it is exposed to the tidal rhythm, intense wave action and sediment instability (de la Huz et al. 2002). In many European regions (Nantón et al. 2017) including Portugal (Gaspar et al. 1999, Pereira et al. 2016), *D. trunculus* constitutes one of the most important artisanal fisheries mainly due to its high economic value. Recorded European landings over the last 10 years were 9,408 tonnes, with a maximum yield of 1,353 t in 2005 (FAO 2006-2018) and although fishing has slightly declined in recent years, as it has been occurring with other clams, namely carpet shells (*Ruditapes decussatus* (Linnaeus, 1758) and *Venerupis corrugata*

(Gmelin, 1791) (FAO 2006-2018)), the wedge clam remains a significant commercial species.

In its habitat, *D. trunculus* population densities are highly variable (Fishelson et al. 1999, Gaspar et al. 1999, Delgado et al. 2017). Besides fishing exploitation, there are many other factors that can control these population densities, including environmental pollution (Fishelson et al. 1999, Neuberger-Cywiak et al. 2003, 2007), seawater acidification (Pereira et al. 2016), temperature (Botelho et al. 2018), sediment grain-size (de la Huz et al. 2002, La Valle et al. 2011) and parasitism. Concerning parasitism, among trematode species infecting the wedge clams, *Bacciger bacciger* (Rudolphi, 1819) Nicoll, 1914 is the most prevalent in European waters (Ramón et al. 1999, de Montaudouin et al. 2014). This parasite has a complex life cycle using three different host species (Palombi 1934). *D. trunculus* is infected by the miracidium free-living stage as the first intermediate host, where the sporocyst parasitic stage develops. From mature sporocysts, cercariae larvae are released in the environment, another free-living form, which rapidly penetrate an amphipod (*Ericthonius brasiliensis* (Dana, 1853)) as second intermediate host where settle as metacercariae. When amphipods are predated by the fish *Atherina* sp., the definitive host, the parasite develops into its adult parasitic stage, sexually reproduces and releases its eggs in the environment. Despite its already studied detrimental effects on wedge clams condition index (de Montaudouin et al. 2014) and reproduction (Ramón et al. 1999), little is known about how this pathogen can affect the health of *D. trunculus* populations and its contribution for the population decline.

The present study aimed to identify the consequences of heavy parasite infection (*B. bacciger* visible sporocysts) in terms of individual response, evaluating the impacts induced in the host health status both at transcriptomic (antioxidant response related genes) and biochemical (metabolic activity, energy reserves, antioxidant activity and cellular damage) levels. The tested hypothesis was that the spread of *B. bacciger*

among tissues will compromise *D. trunculus* regular gene expression and biochemical performance.

2. MATERIAL AND METHODS

2.1 Study area

Donax trunculus were collected in Praia de Faro (Faro's beach) in a single area (37°00' 16''N, 7°59'27''W). Praia de Faro is a na rrow reflective beach (Balouin et al. 2005) located in the Ancão Peninsula of the Ria Formosa coastal lagoon, south of Portugal. This Peninsula is approximately 10 km length and is the most western system of barrier islands of the Ria Formosa. The typical tidal range is between 2 and 3.8 m (Granja et al. 1984). The sampling area is composed mainly of fine (~ 125 µm) sands. The most recent data on Portuguese annual official landings of *D. trunculus* in 2016 were of 252 ton with the sales value of 750 k€ (INE 2018).

2.2 Sampling procedure

Every other month from March 2016 to January 2017 (6 sampling months), *D. trunculus* were collected using a hand-dredger (Figure 1A) which is composed by a rectangular shape metal grid (Figure1B), a 2.8 cm mesh size bag (where the catch is retained) and a fixed wood beam (Figure 1C). Water temperature and salinity were recorded with a multiparametric probe.

In the laboratory, all wedge clams collected in a single haul were measured (± 1 mm) with a calliper and *Bacciger bacciger* prevalence, i.e. the percentage of infected hosts, calculated with two different methods. The two hundred largest organisms were individually placed in plastic containers filled with seawater extracted directly from the sampling area. After 12 hours, these containers were analysed under a stereomicroscope in order to check the presence of free *B. bacciger* cercariae emitted by the wedge clams in the water (this methodology was adapted from Jensen et al. 1999, de Montaudouin et al. 2016 and Magalhães et al. 2017). *B. bacciger* prevalence was calculated considering parasitized clams (P), those surrounded by free cercariae, and non-emitting clams, those with no cercariae in the surrounding water, here

identified as emergence prevalence. Then, fifty wedge clams (from those found negative for the presence of *B. bacciger* cercariae) were opened and squeezed between two glass slides to check parasite presence (this methodology was adapted from de Montaudouin et al. 2000). *B. bacciger* prevalence was further calculated considering P clams, those that under stereomicroscope identification were positive for *B. bacciger* sporocysts presence, and non-parasitized (NP) clams, those that under a stereomicroscope were negative for *B. bacciger* sporocysts presence, identified as dissection prevalence.

Two *D. trunculus* specimens were randomly chosen and dissected to extract gills and digestive gland. These tissue samples were conserved in Ambion™ RNA later® (500 µL) at -80 □C prior to molecular cloning, consequent interest genes isolation and sequences determination.

For transcriptomic analysis, all wedge clams collected (excluding those found positive for *B. bacciger* cercariae emergence and the fifty clams opened) were opened and observed to check *B. bacciger* presence by the naked eye (orange sporocyst mass). The first five wedge clams found negative and positive for *B. bacciger* (a total of 10 samples whenever possible) were immediately transferred into 500 µL of Ambion™ RNA later® and conserved at - 80 ℃.

For biochemical analyses, all the wedge clams left, as well as those found positive for *B. bacciger* cercariae emergence were preserved at - 80 ℃. Wed ge clams were then quickly opened to check *B. bacciger* presence (orange mass) and pooled in groups of 2 accounting for 10 replicates, whenever possible, by condition (parasitized with *B. Bacciger*, P and non-parasitized with *B. bacciger*, NP) and by sampling month.

2.3 Transcriptomic analysis

2.3.1 Total RNA extraction and reverse transcription

All *D. trunculus* samples (obtained and preserved as mentioned in the previous section) were homogenised at room temperature and 40 mg per individual were used.

Total RNAs were extracted using SV Total RNA Isolation System kit (Promega) and reverse transcribed using oligo dT and random primers with the GoScript Reverse Transcription System kit (Promega), according to manufacturer instructions. The concentration of total RNAs was determined spectrophotometrically at 260 nm and purity checked by the 260/280 nm ratio.

2.3.2 PCR and molecular cloning

The cDNAs were amplified using the specific primers. The amplification program consisted of 40 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min and a final elongation step of 72 ℃ for 10 min. Amplif ied products were analysed on one percent agarose gels and fragments of the expected sizes were excised and purified using Wizard SV Gel and PCR Clean-Up System (Promega), according to manufacturer instructions. The resulting products were commercially sequenced (Sigma-Aldrich) and submitted to the GenBank. Partial cDNA sequence of *ef1α*, *β actin*, *Sod (Mn)* and *Cat* genes were successfully isolated using primers derived from conserved regions of the respective sequences.

2.3.3 Primer design

With the aim of recognizing *B. bacciger* effect on the wedge clam cell basic functions, two target genes were chosen: *Cat* and *Sod (Mn)* involved in the oxidative stress response. *Ef1α* and *β actin* were chosen as reference genes. Gene sequences from close related species, available in databases, were aligned using the Clustal Omega free software. From this alignment and for each gene, one forward and one reverse primer were deduced in conserved regions. All primer pairs used in this study are listed in Table 1.

2.3.4 Real-time quantitative PCR

Real-time PCR reactions were performed in a Lightcycler (Bio-rad CFX connect). The amplification program consisted of one cycle at 95 ℃ for 10 min and 50 amplification cycles at 95 ℃ – 5 s, 60 ℃ – 5 s, 7 2 ℃ – 20 s. Each reaction contained

17 µL of master mix including the SYBRgreen I fluorescent dye (Promega), 2 µL of the gene specific primer pair (final concentration 300 nM for each primer) and 1 µL of cDNA. Primers pairs were designed using the Primer 3 plus free software.

The relative quantification of each gene expression level was normalised according to the reference genes and generated using the $2^{-\Delta CT}$ method described by Livak and Schmittgen (2001) where ΔCT represents the difference between the cycle threshold of a specific gene and the cycle threshold of the reference genes. The inductor factor (IF) of each gene was determined in comparison with control (= without *B. bacciger*) corresponding to the following equation (Paul-Pont et al. 2010):

$$IF = \frac{2^{-\Delta CT\ (with\ B.\ bacciger)}}{2^{-\Delta CT\ (without\ B.\ bacciger)}}$$

2.4 Biochemical analyses

Each replicate was homogenised with liquid nitrogen and separated into 0.3 g of soft tissue subsamples in order to perform the extraction with three different buffers. The supernatant of the subsample extracted with phosphate buffer (1:2 w/v) and centrifuged at 4 °C, 10000 *g* during 20 min was used to determine glycogen (GLY) and protein (PROT) concentrations, superoxide dismutase (SOD) and catalase (CAT) activities. The supernatant of the subsample extracted with 0.1 M Tris-HCl (pH 8.5), 15% (w/v) Poly Vinyl Pyrrolidone, 153 µM $MgSO_4$ and 0.2% (w/v) Triton X-100 buffer and centrifuged at 4 °C, 3000 *g* during 20 min was used to determine electron transport system (ETS) activity. The supernatant of the subsample extracted with 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 4 °C, 10000 *g* during 20 min was used to determine lipid peroxidation (LPO) levels. All supernatants were then preserved at - 20 °C or used immediately.

ETS activity is used as a metabolic capacity measure. Its activity was determined by the amount of formazan formed after adding p-IodoNitroTetrazolium (De

Coen and Janssen 1997), calculated using ε = 15.9x10^3 M^{-1} cm^{-1} and expressed in nmol of formazan formed per min per g of fresh weight (FW).

GLY was quantified by the phenol–sulphuric acid method described by Dubois et al. (1956). Absorbance was measured at 492 nm and results were expressed in mg per g FW.

PROT content was determined according to Robinson and Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0 – 40 mg mL^{-1}). After 10 min incubation at 30 ℃ the absorbance was read at 540 nm. The results were expressed in mg per g of FW and used to calculate enzymes activity.

SOD activity was measured using the method described by Beauchamp and Fridovich (1971). The standard curve was determined with SOD standards (0.25 – 60 U mL^{-1}). After 20 min in an orbital incubator set at room temperature, the enzyme activity was measured spectrophotometrically at 560 nm and expressed in unit of enzyme (U) per mg of protein. One U corresponds to a reduction of 50% of Nitro blue tetrazolium (NBT).

CAT activity was measured by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson and Borg 1988). The standard curve was determined using formaldehyde standards (0 – 150 mM). After 20 min in an orbital incubator at room temperature, the formaldehyde formation in the presence of Purpald was spectrophotometrically measured at 540 nm. The enzymatic activity was expressed in U per mg of protein. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde, per min.

LPO was measured by the quantification of thiobarbituric acid reactive substances (TBARS), according to Buege and Aust (1978) protocol. This methodology is based on the reaction of LPO by-products, namely malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) forming TBARS. The amount of MDA was quantified spectrophotometrically and measured at a wavelength of 532 nm (ε = 156mM^{-1} cm^{-1}). Results were expressed as nmol of MDA equivalents per g FW.

2.5 Data analysis

One-way ANOVA, followed by a Tukey post hoc analysis, was used to test differences between mean shell length of the dissected wedge clams used to calculate *B. bacciger* prevalence per sampling month. Correlation between water temperature and *B. bacciger* prevalence was tested using nonparametric Spearman analysis. One-way ANOVAs were performed and, whenever significant, followed by a post hoc analysis (Tukey test) for each gene expressed in order to test differences among sampling months in terms of induction factor. One-way ANOVAs were performed and, whenever significant, followed by a post hoc analysis (Tukey test) for each biochemical marker in order to test: 1) differences among sampling months separately for NP and P clams; 2) differences between NP *and* P clams for each sampling month. For all parametric tests, homogeneity of variances was checked with Cochran test while normality was assumed. Three Principal Coordinates Ordination analysis (PCO) were separately performed for non-parasitized clams, parasitized clams and parasitized vs. non-parasitized clams. PCOs were based on biomarkers data matrices, containing all sampling months. Prior to visualization of the distance among centroids (i.e. the mean position of all the points representing a given sample) on PCO, data were normalised and the Euclidean distance calculated. In the PCO graphs, the variables (biomarkers) that better explained ($r > |0.7|$) the samples spatial distribution were represented as superimposed vectors.

3. RESULTS

3.1 Infection of *Donax trunculus*

Shell length of sampled wedge clams varied between 15 and 40 mm with a frequency peak at 25 mm (Figure 2). Regarding dissected clams, mean shell length was significantly higher in July, September and January (mean ± standard deviation = 32.6 ± 1.9, 33.2 ± 2.0 and 32.6 ± 1.6 mm, respectively) comparing to March, May and

November (29.0 ± 2.9, 24.3 ± 2.9 and 28.9 ± 3.3 mm, respectively) (One-way ANOVA: $F_{(5)} = 91.2$, $p < 0.01$).

Only one macroparasite species was found, the trematode *Bacciger bacciger* identified by the naked eye as an orange mass invading all wedge clam tissues and morphologically verified at the stereomicroscope following Ramón et al. (1999) description.

The dissection prevalence mean value (11.9 ± 10.7) was significantly higher than the mean emergence prevalence (1.3 ± 1.9). Total prevalence (considering infections detected by dissection and by emergence) showed a seasonal trend with the highest value in July (33%) and the lowest in May (0%) (Figure 3). Water temperature showed to be positively correlated to *B. bacciger* total prevalence ($r = 0.83$, $p < 0.05$, Figure 3).

3.2 Transcriptomic data

In July and September, the expression of *Sod* (*Mn*) gene was inhibited in parasitized compared to non-parasitized clams. In March, this gene was over-expressed in parasitized compared to non-parasitized clams (Table 2).

Cat gene was inhibited in July and induced in November for parasitized compared to non-parasitized clams (Table 2).

3.3 Biochemical data

For all biochemical parameters analysed there are no data concerning parasitized clams in March and May due to very low *B. bacciger* total prevalence found in these months.

Regarding ETS, in NP clams, the activity was significantly higher from March to July and significantly lower from September to January, while for P clams ETS activity was higher in July and January compared to September and November (Figure 4A). In July and January, ETS activity was significantly higher in P compared to NP clams (Figure 4A).

GLY content of the NP clams was significantly higher in March and July compared to the other months, and a similar trend (higher GLY content in July because there were no P clams in March) was observed for the P clams (Figure 4B). Overall, there was lower GLY content in P compared NP clams (significant in July and November) (Figure 4 B). PROT content was not significantly different among all conditions (data not shown).

Taking into account NP wedge clams, results showed significantly higher SOD activity in September and significantly lower activity in January, with the remaining months displaying intermediate values (Figure 4C). Regarding P clams, significantly higher SOD activity was presented in November and lower in January (Figure 4C). The global trend showed lower SOD in P compared to NP but only significant in September (Figure 4C).

CAT activity for NP clams was significantly higher in September compared to the other sampling months (Figure 4D). Parasitized clams showed significantly lower CAT activity in November compared to the other sampling months (Figure 4D). The global trend showed lower CAT values in P clams compared to NP, with statistical significant in November (Figure 4D).

For NP clams, LPO values were significantly higher in March and May and significantly lower in November and January compared to the other months, while P clams showed slightly higher LPO in January (Figure 4E). Significant differences were observed between P and NP clams in July and January with lower and higher LPO levels for P clams, respectively (Figure 4E).

The horizontal dimension (Axis 1) of the PCO with NP clams explained 57% of the total variation separating March, May and July, in the negative side of the axis, from September, November and January, in the positive side of the axis. ETS, GLY and LPO biomarkers were the variables that better explained this variation presenting high negative correlation with axis 1 ($r > |0.8|$) as well as CAT activity that presented high positive correlation with axis 1 ($r > |0.7|$). Axis 2 explained 28% of the total variation

which separated mainly January, in the positive side of the axis, from September with a strong positive correlation with SOD activity (r > |0.8|) (Figure 5A).

The axis 1 of the PCO representing the P samples explained 70% of the total variation separating November, in the negative side of the axis, from July and January, in the positive side of the axis. SOD presented high negative correlation (r > |0.8|) and ETS, CAT and LPO biomarkers high positive correlation (r > |0.8|) with axis 1 and together were the variables that better explained the samples separation. Axis 2 explained 26% of the total variation separating January, in the negative side of the axis, from July with a strong positive correlation with GLY content (r > |0.8|) (Figure 5B).

The axis 1 of the PCO concerning P vs. NP samples explained 48% of the total variation separating mainly P clams of November but also NP clams of November and January and P clams of September in the negative side of the axis, from the other conditions in the positive side of the axis. ETS, CAT and LPO biomarkers were the variables that better explained the variation, presenting high positive correlation (r > |0.8|) with axis 1. The PCO vertical dimension (Axis 2) explained 25% of the total variation separating mainly NP clams of September but also NP clams of November, in the positive side of the axis, from the other conditions with a strong positive correlation with SOD activity (r > |0.8|) (Figure 5C).

4. DISCUSSION

Studies of first host – parasite systems and consequent predictions of disease events are very difficult to accomplish mostly due to generally low prevalence of these parasites and/or sudden high prevalence outbreaks that are followed by mass mortalities (Thieltges et al. 2008, Magalhães et al. 2015). The effects of the sporocyst, the parasitic stage occurring in the first intermediate host, are often severe and negative impacts are described at the reproduction (Carballal et al. 2001), growth (Bowers 1969) and behaviour (Babirat et al. 2004) levels. Recently, it has been demonstrated that sporocyst can even interfere with host metabolism, energy reserves and antioxidant response (Magalhães et al. 2017). Particularly for the *Donax trunculus*

– *Bacciger bacciger* host-parasite relationship, it was already demonstrated that this parasite is able to damage the connective and foot tissue of the wedge clam (Ramadan and Ahmad 2010), leading to castration (Ramón et al. 1999) and decreasing its overall condition index (de Montaudouin et al. 2014). However, the present work increased considerably the knowledge in this field by: (1) primarily reporting the first record of *B. bacciger* infecting wedge clams in Portugal, expanding further west the known distribution boundary of this species (Margolis and Ching 1965) previously located in Mehdia, Morocco, and also (2) by representing the first effort to recognise the *B. bacciger* effects on *D. trunculus* gene expression and biochemical markers, over a year monitoring considering seasonal changes.

As previously demonstrated by other authors studying other 1st host-parasite systems (Curtis and Hubbard 1990, Magalhães et al. 2017), our findings demonstrated that prevalence calculated after host dissection was higher than prevalence calculated by cercariae emergence. This difference is due to parasite development, which can be already in the sporocyst form, i.e. visible at the stereomicroscope, but still not mature and so with no cercariae emergence. Therefore, we recommend dissection of the hosts as an important requirement for the correct prevalence calculation.

Overall, *B. bacciger* total (i.e. considering both methods) prevalence (mean = 13%) was similar to values found in Mehdia, Morocco (6.6%) (de Montaudouin et al. 2014), Cullera, Spain (8.4%) (Ramón et al. 1999) and Biscarosse, France (17.7%) (de Montaudouin et al. 2014), in Port Said, Egypt prevalence values were higher (73.7%) (Ramadan and Ahmad 2010). Total prevalence of infection showed a seasonal pattern being highest in July (33%) and lowest in May (0%) as opposite to what was observed in Spain (Ramón et al. 1999, Delgado and Silva 2018). Knowing that generally the prevalence of trematodes infecting bivalves as first intermediate host is positively correlated to host size (Magalhães et al. 2015), the observed seasonal pattern could be related to the shell length confounding factor. Despite the clams sampled in May (*B. bacciger* total prevalence = 0%) have been smaller than clams sampled in July (*B.*

bacciger total prevalence = 33%) conversely, there were other months displaying low prevalence values in large organisms (e.g. January) excluding this feature as a prevalence explanatory variable.

In the present study, the most likely driver of the infection was the water temperature (as proxy of seasonality), a known trigger of trematode infection (de Montaudouin et al. 2016) which showed to be positively correlated with total prevalence. A similar trend was described by Ramadan and Ahmad (2010) studying the same host-parasite system in the north-east coast of Egypt. Seasonality in trematodes infecting their first intermediate host is not frequently observed, mainly due to the natural low prevalence of these parasites and consequent difficulty to find infected hosts (Magalhães et al. 2015). Nevertheless, most of the authors tend to converge by identifying summer and the positively correlated high temperatures as the season presenting the highest prevalence (demonstrated by Bowers (1969) in South Wales and by Desclaux et al. (2002) in France both studying *Cerastoderma edule* (Linnaeus, 1758) infected by *Bucephalus minimus* (Stossich, 1887) Nicoll, 1914).

As mentioned above, negative effects of *B. bacciger* on host health has been described by some authors, including impacts on reproduction (Ramón et al. 1999, Delgado and Silva 2018), soft tissues integrity (Ramadan and Ahmad 2010) and condition index (de Montaudouin et al. 2014). However, the present work represents the first assessment of *B. bacciger* effects at the level of gene expression and biochemical alterations. During an infection, host cells enhance reactive oxygen species (ROS) (Soudant et al. 2013). However, ROS have also important functions in some intracellular signalling cascades activation such as immunity response (Limón-Pacheco and Gonsebatt 2009). Nevertheless, when ROS production exceeds ROS elimination capacity, cellular damage may occur. Therefore, organisms need to balance ROS quantity and for that they mainly use antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) (Regoli and Giuliani 2014). In accordance with such considerations, previous studies with a dinoflagellate parasitized

by *Amoebophrya* (Lu et al. 2016) showed upregulation of genes involved in the calcium signalling which in turn is related to the stress response. Furthermore, studying the disk abalone (*Haliotis discus discus*) challenged by bacteria and viruses, De Zoysa et al. (2011) showed upregulation of the glutathione peroxidase gene. Similarly, glutathione S-transferases genes were upregulated in *Ruditapes decussatus* clams infected by *Perkinsus* sp. (Leite et al. 2013). All these genes are related to the antioxidant system, which is responsible for ROS elimination (Regoli and Giuliani 2014). Our results showed that *Sod (Mn)* and *Cat* oxidative stress related genes were upregulated in clams infected by *B. bacciger* in March (*Sod (Mn)*) and in November (*Cat*), while in July and September the same genes were downregulated in parasitized clams. These results may be explained by the fact that upregulation could represent a response to more than one stressful condition occurring at the same time. Particularly for the present case study, the combination of parasite infection and the peak of the spawning period occurring in March (Gaspar et al. 1999) and the combination of parasite infection and gametogenesis starting in November (Gaspar et al. op. cit). On the other hand, the expression of genes related to the antioxidant defense may have been modulated by the parasite patency. An infection is considered 'patent' when direct evidence of the parasite can be detected and so it is related to the development of the sporocyst in the host (Graczyk and Fried 2007). In March, the visible *B. bacciger* total prevalence was still low (2%), although it was already possible to observe a sporocyst (i.e. prepatent infection). In July and September, the highest *B. bacciger* total prevalence values were registered (33 and 21%, respectively) with mature sporocysts, and cercariae (patent infection). In November, the parasite returns to a prepatent infection. Following these arguments, downregulation of *Sod (Mn)* and *Cat* occurring when sporocysts were mature, can indicate the initiation of an alternative immune or stress response induced by higher parasite spread and consequent pathogenicity or an overall loss of ROS arrest signalling.

Taking only into account non-parasitized clams (sampled in all sampling months) and regarding biochemical parameters, these showed to be responsive to the seasonal changes occurring at both biotic and abiotic levels. The metabolic rate (measured through ETS activity) and energy reserves (GLY content) registered a similar pattern with the highest values in March, May and July, overlapping the entire spawning period and the warmer seasons (spring and summer), and the lowest values in September, November and January, the post-spawning period followed by the colder season. Gametogenesis and spawning periods (frequently occurring during the warmer seasons) are usually characterized by higher metabolism demand. During these periods, GLY is an important energy source, which usually results in a higher condition index (Singh 2017). The same seasonal trend in terms of energy reserves was identified in a *D. trunculus* population from Tunisia (Tlili et al. 2013). After the spawning period and during winter, a decrease in metabolic activities is probably a consequence of energy levels recovery after this reproduction process combine with adverse environmental conditions such as low temperature, low salinity and less quality and/or quantity of food. Previous studies already demonstrated that winter conditions tend to reduce the wedge clams fat content (Özden et al. 2009) and condition index (Tlili et al. 2011). As it was reported for *R. philippinarum* (Anacleto et al. 2014) this decrease of metabolic rate and food intake could be explained by the bivalves strategy of closing their valves when under stressful conditions in order to enhance survival. Overall, higher antioxidant activity was registered in September (SOD and CAT activities) compared to the other sampling months corresponding to a post-spawning period, a stressful period, when *D. trunculus* condition index used to reach its lowest values (Gaspar et al. 1999). The described seasonal trend of metabolic rate and energy reserves (higher in March, May and July comparatively to September, November and January) as well as the relatively lower antioxidant activity resulted in a similar cellular damage trend.

When *B. bacciger* infection was observed (from July to January), this parasite showed to modulate the clam biomarker response changing the seasonal pattern described above. Assessed biomarkers showed to be responsive not only to the individual and environmental seasonal changes but also to the parasite infection. Parasitized clams showed higher metabolic rate (measured here by ETS activity) and lower GLY content. Correspondingly, the gastropod *Lymnaea stagnalis* infected with trematodes, showed an exhaustion of energy-cell resources experienced by the host which led to a decrease in CAT activity (Khomich et al. 2017). An infected clam has an additional metabolic requirement having to supply its own survival but also parasites with sufficient energy to grow (MacLeod 2017). Overall, *B. bacciger* infection inhibited the SOD and CAT activities, which is in agreement with the reduced *Sod (Mn)* and *Cat* expression registered in July and September. Similarly, *Clinostomum detruncatum* (trematode) infection proved to reduce the non-enzymatic antioxidant defenses with respect to pro-oxidant status in the muscle of the freshwater teleost *Rhamdia quelen* (Belló et al. 2000). Moreover, some vibrio species, such as *Vibrio tapetis*, shown to be able to inhibit reactive oxygen intermediates production (such as hydrogen peroxide) in hemocytes of *Crassostrea gigas* (Lambert et al. 2003). Transcriptional and biochemical results were not convergent in January, since *Sod (Mn)* and *Cat* genes were upregulated in parasitized clams but measured SOD and CAT activities were lower. Straight correlations between mRNA expression and the respective enzyme activity are still poorly studied and with controversial results but, certainly not always positively correlated and dependent on post-transcriptional, translational, regulation and protein degradation related processes (Vogel and Marcotte 2012). Finally, especially noticed in January, the increased metabolism and the reduction in the antioxidant enzymes activity led to oxidative stress (higher LPO level) and consequently to cellular damage in parasitized clams.

5. CONCLUSIONS

Overall, the present study showed that *B. bacciger* has a negative effect on its first intermediate host *Donax trunculus,* by increasing its metabolic rate, decreasing the energy reserves and inhibiting the antioxidant enzymes activity, which in some months led to cellular damage (measured by LPO levels). Our findings showed that transcriptomic and biochemical markers can provide additional and ecologically relevant information regarding parasite effects on their hosts. Hence, these markers can not only reflect the environmental conditions that animals experience but also the invasion effects of pathogens, helping to predict the organism chance of reproduction and survival in their natural context. This approach can therefore help conservation practitioners to identify conservation threats to bivalve populations and to maximize the success of stock and disease episodes management. Moreover, the present study showed the importance of parasitology integration into physiological assessment of marine organisms exposed to stressful conditions to avoid incorrect identification of marine species as tolerant or susceptible to a given stress, when in fact the physiological response of the organism is modified by parasitic infection.

Acknowledgements

Luísa Magalhães benefited from a PhD grant (reference: PD/BD/52570/2014) given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) and European Union. Simão Correia benefited from a research fellowship funded by INTERREG-ATLANTIC program through the research project COCKLES (EAPA_458/2016 COCKLES Co-Operation for Restoring CocKle SheLlfisheries & its Ecosystem-Services in the Atlantic Area). Rosa Freitas benefited from a research position funded by the Integrated Programme of SR&TD “Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate” (reference: Centro-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund. Thanks are also due to CESAM

(UID/AMB/50017) and to FCT/MEC, through national funds, for the financial support and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

Authors are grateful to Isidoro Costa for collecting *Donax trunculus* specimens and to Carlos Afonso for helping with CCMAR lab facilities and work logistic.

References

Anacleto, P., A. L. Maulvault, V. M. Lopes, T. Repolho, M. Diniz, M. L. Nunes, A. Marques, and R. Rosa. 2014. Ecophysiology of native and alien-invasive clams in an ocean warming context. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 175:28-37.

Babirat, C., K. N. Mouritsen, and R. Poulin. 2004. Equal partnership: two trematode species, not one, manipulate the burrowing behaviour of the New Zealand cockle, *Austrovenus stutchburyi*. Journal of Helminthology 78:195-199.

Balouin, Y., H. Howa, R. Pedreros, and D. Michel. 2005. Longshore sediment movements from tracers and models, Praia de Faro, South Portugal. Journal of Coastal Research 21:146-156.

Bayed, A., and J. Guillou. 1985. Contribution à l'étude des populations du genre *Donax*: La population de *D. trunculus* L. (Mollusca, Bivalvia) de Mehdia (Maroc). Annales de l'Institut océanographique 61:139-147.

Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44:276-287.

Belló, A. R. R., E. Fortes, A. Bello-Klein, A. A. Bello, S. F. Llesuy, R. B. Robaldo, and A. Bianchini. 2000. Lipid peroxidation induced by *Clinostomum detruncatum* in muscle of the freshwater fish *Rhamdia quelen*. Diseases of Aquatic Organisms 42:233-236.

Botelho, M. J., C. Vale, S. Joaquim, S. T. Costa, F. Soares, C. Roque, and D. Matias. 2018. Combined effect of temperature and nutritional regime on the elimination of the lipophilic toxin okadaic acid in the naturally contaminated wedge shell *Donax trunculus*. Chemosphere 190:166-173.

Bowers, E. A. 1969. Cercaria *Bucephalopsis haimeana* (Lacaze-Duthiers, 1854) (Digenea: Bucephalidae) in cockle, *Cardium edule* L. in south Wales. Journal of Natural History 3:409-422.

Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. Methods in enzymology 52:302-310.

Carballal, M. J., D. Iglesias, J. Santamarina, B. Ferro-Soto, and A. Villalba. 2001. Parasites and pathologic conditions of the cockle *Cerastoderma edule* populations of the coast of Galicia (NW Spain). Journal of Invertebrate Pathology 78:87-97.

Curtis, L. A., and K. M. Hubbard. 1990. Trematode infections in a gastropod host misrepresented by observing shed cercariae. Journal of Experimental Marine Biology and Ecology 143:131-137.

De Coen, W. M., and C. R. Janssen. 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. Journal of Aquatic Ecosystem Stress and Recovery 6:43-55.

de la Huz, R., M. Lastra, and J. Lopez. 2002. The influence of sediment grain size on burrowing, growth and metabolism of *Donax trunculus* L. (Bivalvia: Donacidae). Journal of Sea Research 47:85-95.

de Montaudouin, X., H. Bazairi, K. A. Mlik, and P. Gonzalez. 2014. *Bacciger bacciger* (Trematoda: Fellodistomidae) infection effects on wedge clam *Donax trunculus* condition. Diseases of Aquatic Organisms 111:259-267.

de Montaudouin, X., H. Blanchet, C. Desclaux-Marchand, N. Lavesque, and G. Bachelet. 2016. Cockle infection by *Himasthla quissetensis* - I. From cercariae emergence to metacercariae infection. Journal of Sea Research 113:99-107.

de Montaudouin, X., I. Kisielewski, G. Bachelet, C. Desclaux. 2000. A census of macroparasites in an intertidal bivalve community, Arcachon Bay, France. Oceanologica Acta 23:453-468.

De Zoysa, M., I. Whang, C. Nikapitiya, C. Oh, C. Y. Choi, and J. Lee. 2011. Transcriptional analysis of disk abalone (*Haliotis discus discus*) antioxidant enzymes

against marine bacteria and virus challenge. Fish and Shellfish Immunology 31:155-160.

Delgado, M., and L. Silva. 2018. Timing variations and effects of size on the reproductive output of the wedge clam *Donax trunculus* (L. 1758) in the littoral of Huelva (SW Spain). Journal of the Marine Biological Association of the United Kingdom 98:341-350.

Delgado, M., L. Silva, S. Gomez, E. Masferrer, M. Cojan, and M. B. Gaspar. 2017. Population and production parameters of the wedge clam *Donax trunculus* (Linnaeus, 1758) in intertidal areas on the southwest Spanish coast: Considerations in relation to protected areas. Fisheries Research 193:232-241.

Desclaux, C., X. de Montaudouin, and G. Bachelet. 2002. Cockle emergence at the sediment surface: 'favourization' mechanism by digenean parasites? Diseases of Aquatic Organisms 52:137-149.

Deval, M. C. 2009. Growth and reproduction of the wedge clam (*Donax trunculus*) in the Sea of Marmara, Turkey. Journal of Applied Ichthyology 25:551-558.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356.

FAO 2006-2018. Fisheries and aquaculture software. FishStat Plus - Universal software for fishery statistical time series. FAO Fisheries and Aquaculture Department [online], Rome.

Fishelson, L., V. Bresler, R. Manelis, Z. Zuk-Rimon, A. Dotan, H. Hornung, and A. Yawetz. 1999. Toxicological aspects associated with the ecology of *Donax trunculus* (Bivalvia, Mollusca) in a polluted environment. Science of the Total Environment 226:121-131.

Gaspar, M. B., L. M. Chicharo, P. Vasconcelos, A. Garcia, A. R. Santos, and C. C. Monteiro. 2002. Depth segregation phenomenon in *Donax trunculus* (Bivalvia:

Donacidae) populations of the Algarve coast (southern Portugal). Scientia Marina 66:111-121.

Gaspar, M. B., R. Ferreira, and C. C. Monteiro. 1999. Growth and reproductive cycle of *Donax trunculus* L., (Mollusca: Bivalvia) off Faro, southern Portugal. Fisheries Research 41:309-316.

Graczyk, T. K., and B. Fried. 2007. Human waterborne trematode and protozoan infections. Advances in Parasitology 64:111-160.

Granja, H., J. M. Froidefond, and T. Pera. 1984. Processus d'evolution morpho-sedimentaire de la Ria Formosa (Portugal). Bulletin de l'Institut de géologie du Bassin d'Aquitaine 36:37-50.

INE 2018. Fisheries statistics reports. INE Instituto nacional de estatística, Lisboa, https://www.ine.pt. Accessed on 15th March 2018.

Jensen, K. T., N. F. Castro, G. Bachelet. 1999. Infectivity of *Himasthla* spp. (Trematoda) in cockle (*Cerastoderma edule*) spat. Journal of the Marine Biological Association of the United Kingdom 79:265-271.

Johansson, L. H., and L. A. H. Borg. 1988. A spectrophotometric method for determination of Catalase activity in small tissue samples. Analytical Biochemistry 174:331-336.

Khomich, A. S., D. V. Axenov-Gribanov, O. A. Bodilovskaya, Y. A. Shirokova, E. P. Shchapova, Y. A. Lubyaga, Z. M. Shatilina, V. A. Emshanova, and A. P. Golubev. 2017. Assessment of the joint effect of thermal stress, pollution, and parasitic infestation on the activity of antioxidative enzymes in pulmonate mollusk *Lymnaea stagnalis*. Contemporary Problems of Ecology 10:157-163.

La Valle, P., L. Nicoletti, M. G. Finoia, and G. D. Ardizzone. 2011. *Donax trunculus* (Bivalvia: Donacidae) as a potential biological indicator of grain-size variations in beach sediment. Ecological Indicators 11:1426-1436.

Lambert, C., P. Soudant, G. Choquet, and C. Paillard. 2003. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish and Shellfish Immunology 15:225-240.

Leite, R. B., M. Milan, A. Coppe, S. Bortoluzzi, A. dos Anjos, R. Reinhardt, C. Saavedra, T. Patarnello, M. L. Cancela, and L. Bargelloni. 2013. mRNA-Seq and microarray development for the Grooved carpet shell clam, *Ruditapes decussatus*: a functional approach to unravel host -parasite interaction. Bmc Genomics 14:741.

Limón-Pacheco, J., and M. E. Gonsebatt. 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 674:137-147.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402-408.

Lu, Y. M., S. Wohlrab, M. Groth, G. Glockner, L. Guillou, and U. John. 2016. Transcriptomic profiling of *Alexandrium fundyense* during physical interaction with or exposure to chemical signals from the parasite *Amoebophrya*. Molecular Ecology 25:1294-1307.

MacLeod, C. D. 2017. Parasitic infection: a missing piece of the ocean acidification puzzle. Ices Journal of Marine Science 74:929-933.

Magalhães, L., X. de Montaudouin, R. Freitas, G. Daffe, E. Figueira, and P. Gonzalez. 2017. Seasonal variation of transcriptomic and biochemical parameters of cockles (*Cerastoderma edule*) related to their infection by trematode parasites. Journal of Invertebrate Pathology 148:73-80.

Magalhães, L., R. Freitas, and X. de Montaudouin. 2015. Review: *Bucephalus minimus*, a deleterious trematode parasite of cockles *Cerastoderma* spp. Parasitology Research 114:1263-1278.

Margolis, L., and H. L. Ching. 1965. Review of the trematode genera *Bacciger* and *Pentagramma* (Fellodistomatidae) and description of *P. petrowi* (Layman, 1930) n. comb. from marine fishes from the pacific coast of Canada. Canadian Journal of Zoology 43:381-405.

Nantón, A., A. Arias-Perez, R. Freire, J. Fernandez-Perez, S. Novoa, and J. Mendez. 2017. Microsatellite variation in *Donax trunculus* from the Iberian Peninsula, with particular attention to Galician estuaries (NW Spain). Estuarine Coastal and Shelf Science 197:27-34.

Neuberger-Cywiak, L., Y. Achituv, and E. M. Garcia. 2003. Effects of zinc and cadmium on the burrowing behavior, LC50, and LT50 on *Donax trunculus* Linnaeus (Bivalvia-Donacidae). Bulletin of Environmental Contamination and Toxicology 70:713-722.

Neuberger-Cywiak, L., Y. Achituv, and E. M. Garcia. 2007. Effects of sublethal Zn++ and Cd++ concentrations on filtration rate, absorption efficiency and scope for growth in *Donax trunculus* (Bivalvia; Donacidae). Bulletin of Environmental Contamination and Toxicology 79:622-627.

Özden, O., N. Erkan, and S. Ulusoy. 2009. Seasonal variations in the macronutrient mineral and proximate composition of two clams (*Chamelea gallina* and *Donax trunculus*). International Journal of Food Sciences and Nutrition 60:402-412.

Palombi, A. (1934) *Bacciger bacciger* (Rud.) trematode digenetico: fam. Steringophoridae Odhner. Anatomia, sistematica e biologia. Pubblicazioni della Stazioni Zoologica di Napoli 13:438–478

Paul-Pont, I., P. Gonzalez, M. Baudrimont, F. Jude, N. Raymond, L. Bourrasseau, N. Le Goic, F. Haynes, A. Legeay, C. Paillard, and X. de Montaudouin. 2010. Interactive effects of metal contamination and pathogenic organisms on the marine bivalve *Cerastoderma edule*. Marine Pollution Bulletin 60:515-525.

Pereira, A. M., P. Range, A. Campoy, A. P. Oliveira, S. Joaquim, D. Matias, L. Chicharo, and M. B. Gaspar. 2016. Larval hatching and development of the wedge

shell (*Donax trunculus* L.) under increased CO_2 in southern Portugal. Regional Environmental Change 16:855-864.

Ramadan A. M. R and A. M. Ahmad. 2010. Infestation of *Donax trunculus* (Bivalvia, Donacidae) from Mediterranean Sea at Port Said coastal zone with *Bacciger bacciger* (Trematoda, Fellodistomidae) and the role of the parasite in castration of the host. African Journal of Marine Sciences 6:83-94.

Ramón, M., M. Gracenea, and O. Gonzalez-Moreno. 1999. *Bacciger bacciger* (Trematoda, Fellodistomidae) infection in commercial clams *Donax trunculus* (Bivalvia, Donacidae) from the sandy beaches of the Western Mediterranean. Diseases of Aquatic Organisms 35:37-46.

Regoli, F., and M. E. Giuliani. 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research 93:106-117.

Robinson, H. W., and C. G. Hogden. 1940. The biuret reaction in the determination of serum proteins I. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. Journal of Biological Chemistry 135:707-725.

Singh, Y. T. 2017. Relationships between environmental factors and biological parameters of Asian wedge clam, *Donax scortum*, morphometric analysis, length-weight relationship and condition index: a first report in Asia. Journal of the Marine Biological Association of the United Kingdom 97:1617-1633.

Soudant, P., F. L. E. Chu, and A. Volety. 2013. Host-parasite interactions: Marine bivalve molluscs and protozoan parasites, *Perkinsus* species. Journal of Invertebrate Pathology 114:196-216.

Tebble, N. 1966. British Bivalve Seashells. A Handbook for Identification. Trustees of the British Museum (Natural History), London.

Thieltges, D. W., K. T. Jensen, and R. Poulin. 2008. The role of biotic factors in the transmission of free-living endohelminth stages. Parasitology 135:407-426.

Tlili, S., I. Metais, N. Ayache, H. Boussetta, and C. Mouneyrac. 2011. Is the reproduction of *Donax trunculus* affected by their sites of origin contrasted by their level of contamination? Chemosphere 84:1362-1370.

Tlili, S., L. Minguez, L. Giamberini, A. Geffard, H. Boussetta, and C. Mouneyrac. 2013. Assessment of the health status of *Donax trunculus* from the Gulf of Tunis using integrative biomarker indices. Ecological Indicators 32:285-293.

Vogel, C., and E. M. Marcotte. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics 13:227-232.

Figure captions

Fig. 1 *Donax trunculus* sampling method. A) Fisherman handling the Portuguese hand-dredger; B) Details of the rectangular shape metal grid that composes the hand-dredger; C) General view of the Portuguese hand-dredger.

Fig. 2 *Donax trunculus* shell length histograms showing the sampled cohort.

Fig. 3 Seasonal trend of the *Bacciger bacciger* prevalence checked by two methods (cercariae emergence after 12 h and dissection) along with the mean water temperature (±SD) of the sampled area.

Fig. 4 Mean values (± standard deviation) and significant differences represented with different lower (NP: non-parasitized), upper (P: parasitized) case letters and with asterisk (NP vs. P) of A: ETS, electron transport system activity; B: GLY, glycogen content; C: SOD, superoxide dismutase activity; D: CAT, catalase activity and E: LPO, lipid peroxidation levels in *Donax trunculus* wedge clams non-parasitized (N = 10) and parasitized (Jul: N = 6, Sep: N = 4, Nov: N = 1, Jan: N =1) with *Bacciger bacciger*. nd: no data.

Fig. 5 Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution for A: Non-parasitized (NP) *Donax trunculus* wedge clams, B: Parasitized (P) wedge clams and C: NP vs. P wedge clams.

Table 1 Nucleotide sequences of specific primer pairs used in this study.

Gene	Accession number	Function	Sequence 5'-3'
Ef1α	MK388166	Protein synthesis and degradation (reference gene)	TCCCACTCCAGGACGTTTAC[a] TCCTGGGAGAGCTTCTGGTA[b]
β actin	MK388165	Cell structure (reference gene)	CCCACACCGTACCCATCTAC[a] GGGCAACATAGCAGAGCTTC[b]
Sod (Mn)	MK388163	Oxidative stress (manganese)	GCATCTTCTGGCAAGTCCTC[a] GAGAGCGTCCTGATTTGCTC[b]
Cat	MK388164	Oxidative stress	TGACCAGGGCATTAAGAACC[a] AGCACCATCTTACCCACAGG[b]

Abbreviations: [a]foward primer; [b]reverse primer

Table 2 Comparative basal expression expressed in terms of induction factor for the selected genes from parasitized and control clams (non-parasitized clams).

	Mar	May	Jul	Sep	Nov	Jan
sod (Mn)	4.9	—	0.3	0.3	—	—
cat	—	—	0.5	—	2.5	—

expression not different (—); inhibition (expression ratio < 0.5); induction (expression ratio >2)

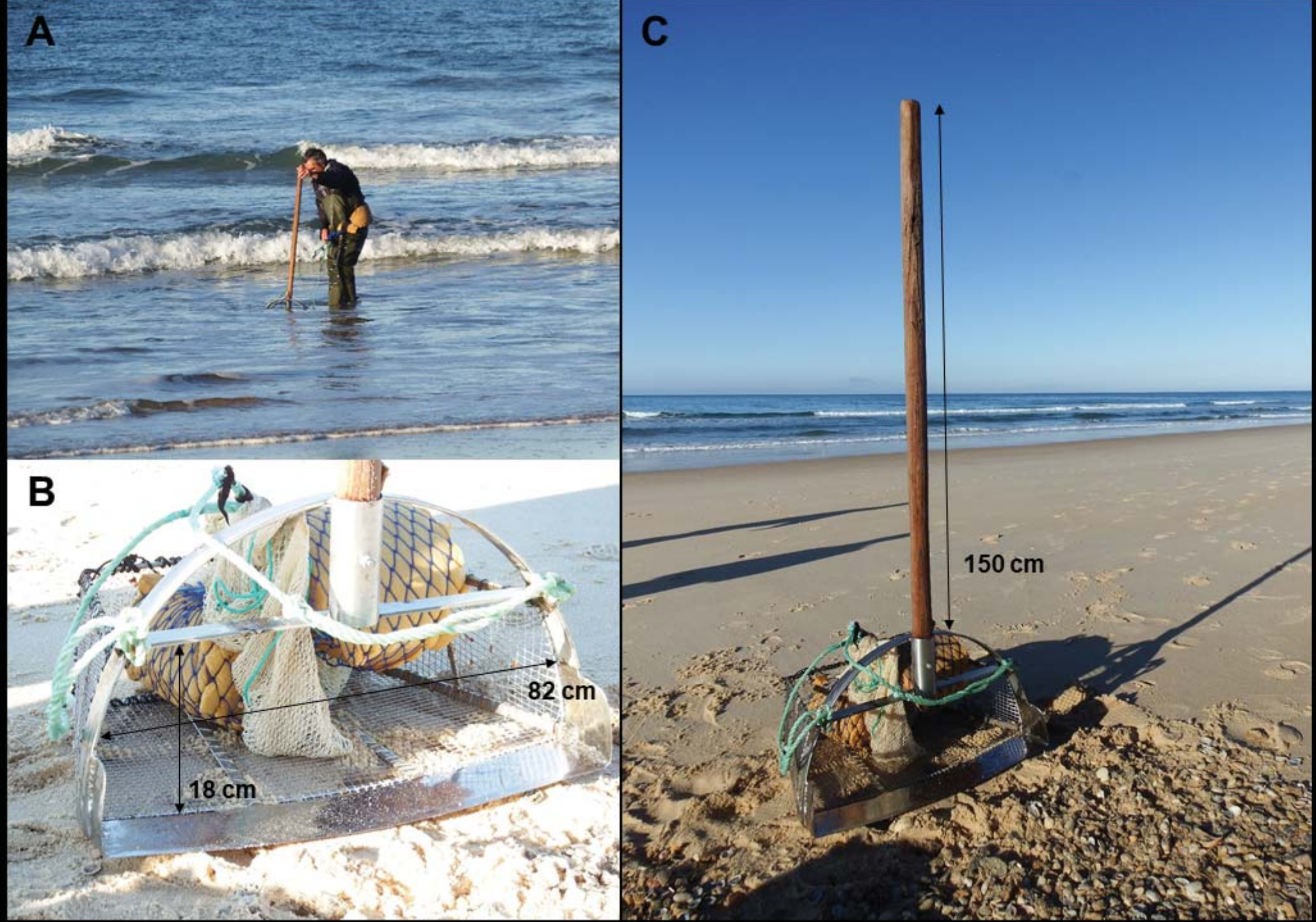

A
B
82 cm
18 cm
C
150 cm

Figure 2

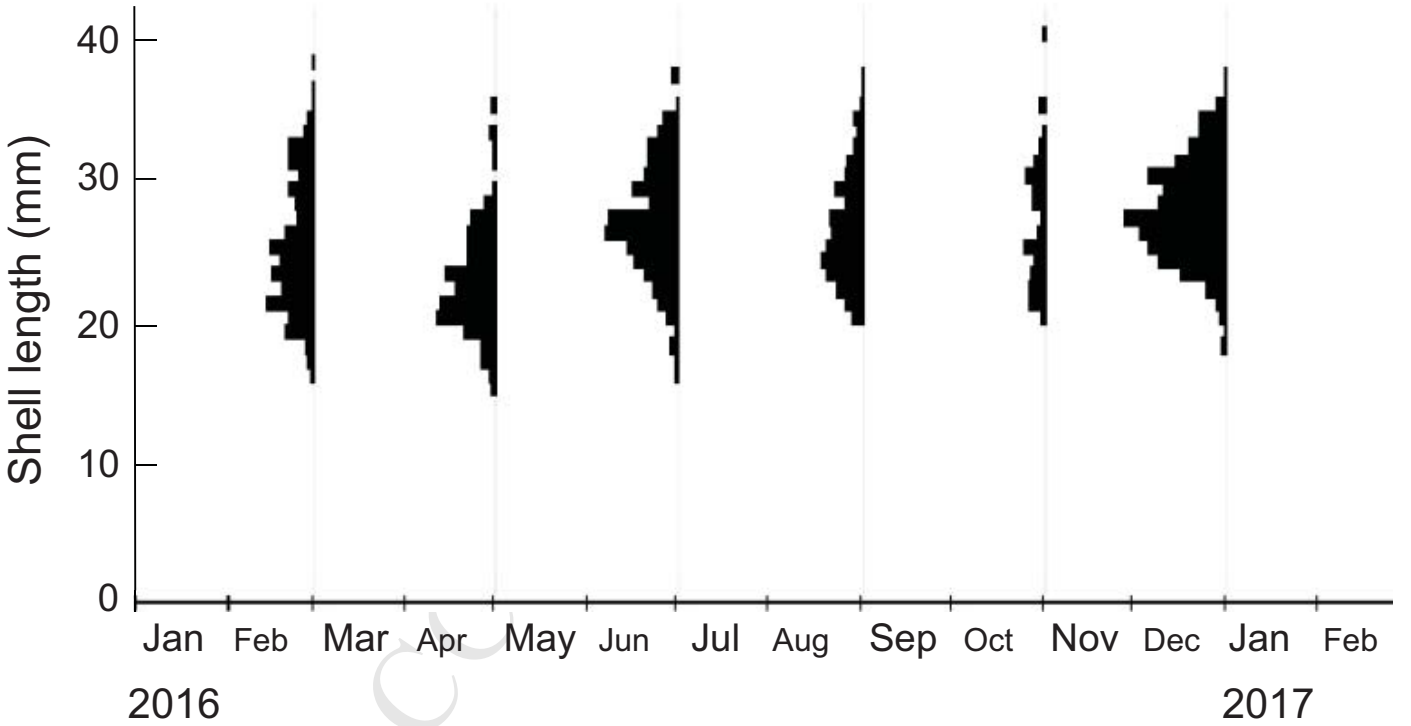

Shell length (mm)
40
30
20
10
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
2016
2017

Figure 3

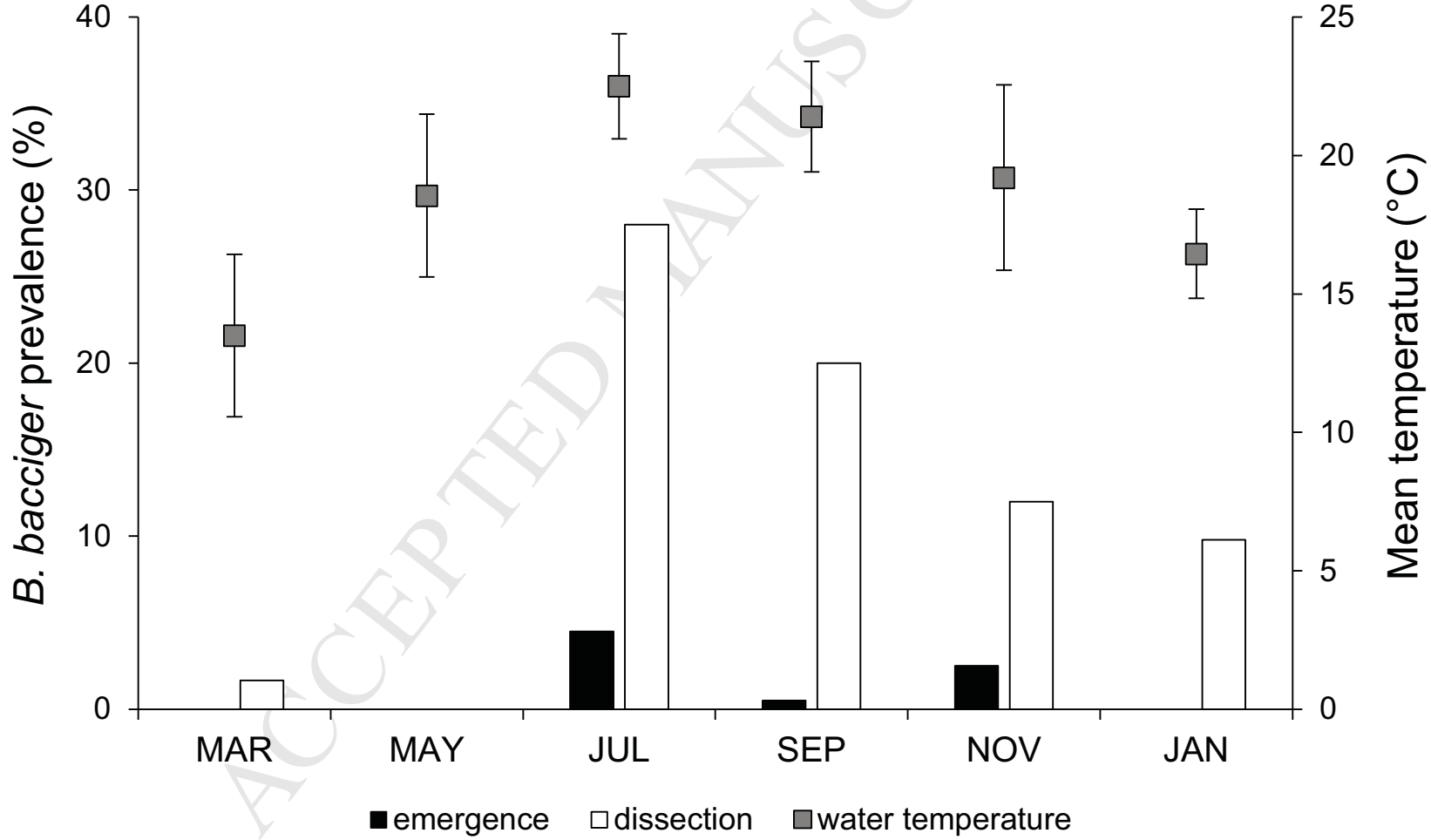


Figure 4

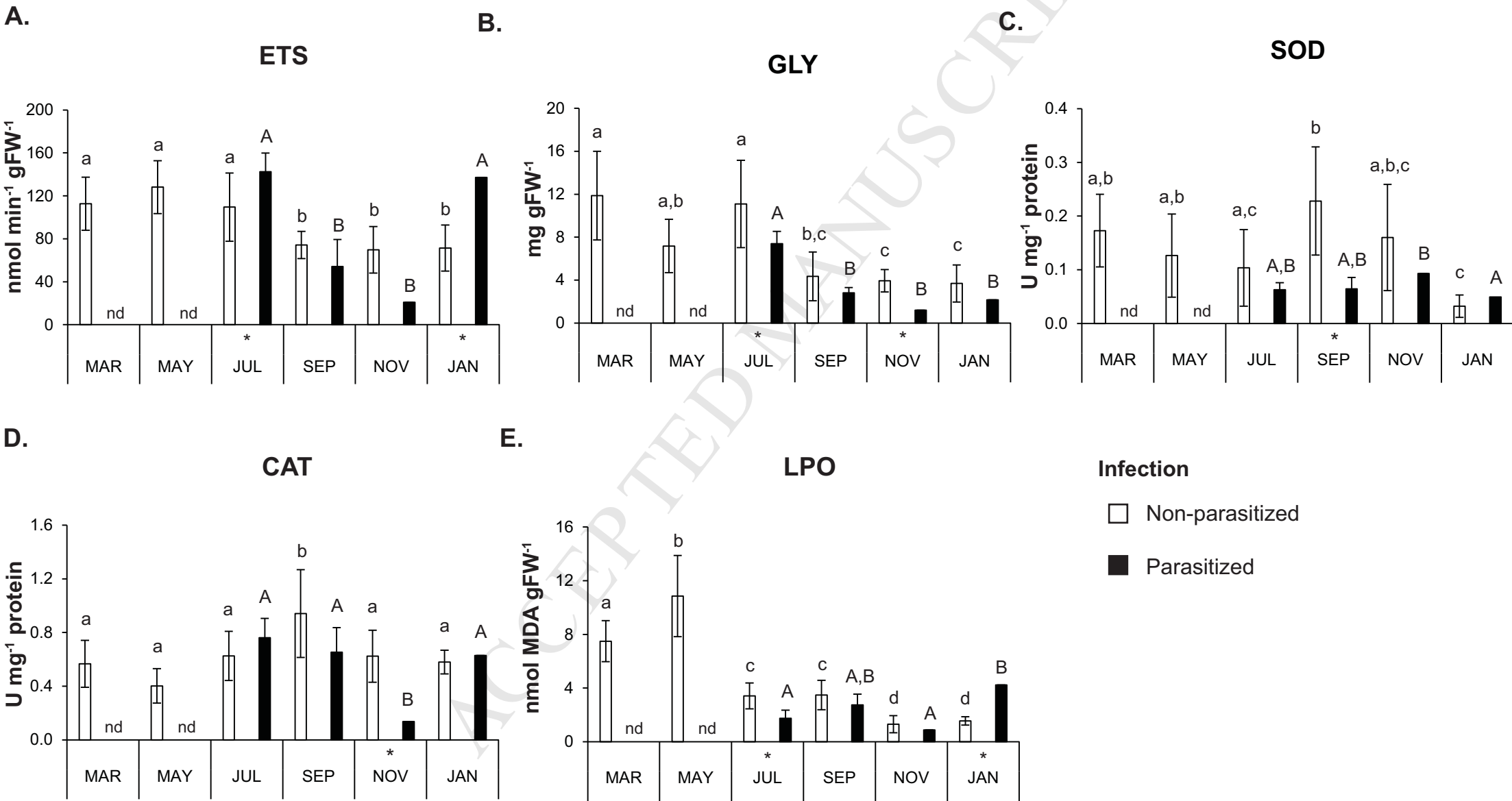
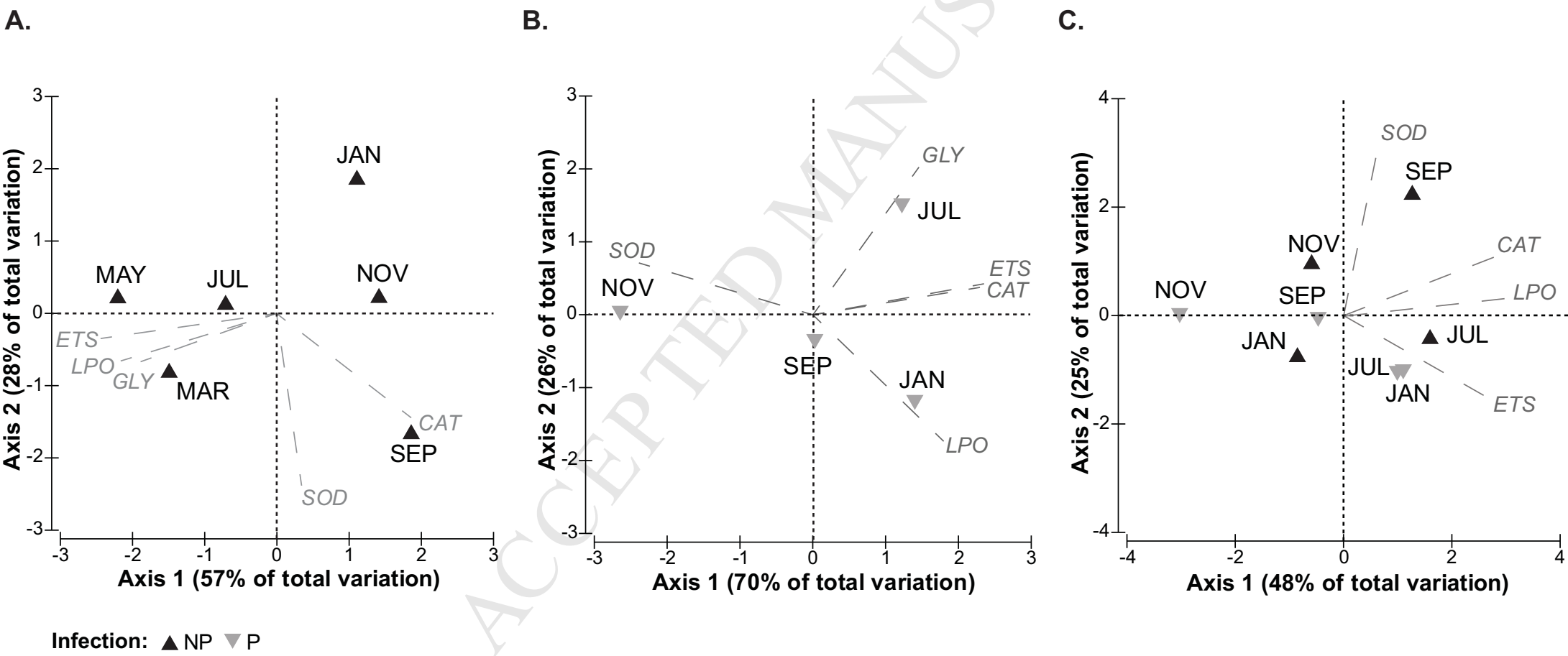


Figure 5



Highlights

- First record of *Bacciger bacciger* infecting *Donax trunculus* in Portugal.
- *Bacciger bacciger* prevalence followed a seasonal pattern.
- *Bacciger bacciger* activated clams defense mechanism against oxidative stress.
- *Bacciger bacciger* increased clams metabolism and energy demand.
- Trematode parasites can interfere with host population performance.